Supplemental Information

Supplemental Experimental Procedures

DNA constructs

Sequence encoding scFv-sfGFP-GB1 was PCR amplified from the published construct obtained from Addgene (Plasmid #60907) and subcloned into lenti-viral vector pFUGW. Sequence encoding NLS-tdPCP-tdTomato was custom synthesized to avoid repeating sequences in tandem PCP and tandem tdTomato, and subcloned into pFUGW. NLS stands for the nuclear localization signal.

Construct containing a tandem (24x) V4 peptide array fused to human ornithine decarboxylase (ODC) and a tandem (24x) PP7 stem-loop array (Figure 1A) was cloned into pEF. In brief, the 24x PP7 fragment was digested from pCR4-24xPP7SL by BamHI and EcoRI, which was then assembled through Gibson assembly (New England Biolabs) with the pEF backbone (Addgene plasmid #11154) to create pEF-24xPP7. The pEF backbone was amplified with primers containing overlapping region with the 24x PP7 fragment to facilitate the assembly. During this process, an EcoRI site and a NotI site was placed between the EF1a promoter and the 24xPP7 sequence. pEF-24xPP7 was then digested with EcoRI and NotI, and the linearized digestion product was assembled by Gibson assembly with the 24xV4 peptide array fragment (from Plasmid #61058 of Addgene) and the ODC fragment (Origene), both PCR amplified with overlapping regions, to create pEF-24xV4-ODC-24xPP7 and pEF-ODC-24xPP7. While pEF-24xV4-ODC is created through assemble of 24xV4 fragment, ODC fragment and pEF backbone. pEF-24xPP7 backbone, 24xV4 peptide array and a maltose binding protein (MBP) with CAAX sequence fused to the C terminus are assembled to created pEF-24xV4-MBP-CAAX-24xPP7.

For experiments using Homo inhibition to measure ribosome elongation rate, to generate open reading frames of different lengths, sequence encoding TagBFP or MBP was inserted between the V4 peptide array and the ODC gene by Gibson assembly of four fragments (pEF-24xPP7 backbone, 24xV4 peptide array, TagBFP or MBP, and ODC).

For experiments with the ATF4 upstream open reading frames (uORFs), we generated the construct pEF-ATF4 uORFs-24xV4-ODC-24xPP7, in which the two ATF4 uORFs (uORF1 and uORF2), from +1 position to the stop codon of the uORF2 (NCBI Accession BC022088.2), was placed upstream of 24x V4 peptide array. We removed the original start codon of the V4 peptide array and placed the peptide array in frame with the start codon of the third ORF (ORF3), which was placed before the stop codon of uORF2. This construct was created by Gibson assembly of three fragments (pEF-24xPP7 backbone, ATF4 uORFs (uORF1 and uORF2), 24xV4 peptide array-ODC).

In the study of ER-targeted protein translation, secreted form of BFP (sBFP) was generated by placing the sequence encoding the hemagglutinin signal peptide upstream of the sequence encoding BFP. The membrane protein construct SMOTM was obtained from a construct used for structural determination of SMO transmembrane domain (Wang et al., 2013). The sequences of these proteins were assembled with the pEF-24xPP7 backbone and the 24xV4 peptide array fused to ODC. A P2A sequence was introduced between sBFP (or SMOTM) and the 24xV4 peptide array.

For neuron studies, 24xV4 peptide array fused to mouse ODC was cloned into pFUGW with or without the 3' untranslated region (UTR) of Arc (NCBI Accession NM_018790.3: position 1390-3038) using Gibson assembly.

Inhibitors

Puromycin dihydrochloride was purchased from Sigma-Aldrich (P9620). Cycloheximide was purchased from Sigma-Aldrich (C1988). Homoharringtonine was purchased from Tocris (1416). GSK 2606414 was purchased from Tocris (5107). ISRIB was purchased from Sigma-Aldrich (SML0843).

Lentivirus production

HEK293 cells (ATCC) were plated at a density of 4-5 million cells per 10 cm dish. One the second day, the cells were transfected with pFUGW encoding construct of interest and helper plasmids VSV-G and delta 8.9 (gifts from Josh Sanes' lab, Harvard University), using Lipofactamine 2000 (Life technologies). The medium was exchanged after 8 h. After 2 days, the medium were collected and filtered with 0.45 μm pore-size filter. The viruses were concentrated using Lenti-X concentrator (Clontech) and kept at -80 °C.

HeLa cell line stably expressing scFv-sfGFP-GB1 and NLS-tdPCP-tdTomato

HeLa cells (ATCC) were maintained in EMEM supplemented with 10% FBS and 1% Pen Strep. To generate a stable cell line to image translation, HeLa cells were transfected by lentiviruses encoding scFv-sfGFP-GB1 and NLS-tdPCP-tdtomato packaged using pFUGW vector. Single clones with different expression levels of fluorescent reporters were sorted by fluorescence activated cell sorting. Each clone was screened for good labeling of translating polysomes by transfection with pEF-24xV4-ODC-24xPP7 and the clone that gave the highest signal-to-noise ratio images of polysomes were used. HeLa cells of the selected clone expressing svFv-GFP and NLS-tdPCP-tdTomato were plated in eight-well chamber (Labtek) one day before transfection of construct of interest. Transient transfection of various translation reporter constructs was performed using lipofectamine LTX (Life technologies) according to the manufacturer's instruction. 12 h after transfection, the medium was exchanged into DMEM supplemented with 10% FBS, 1% Pen Strep, with no phenol-red, buffered by HEPES (pH 7.4), prior to imaging.

Primary hippocampal neuron culture

Primary cultures of mouse hippocampal neurons were prepared using a protocol as previously described (Xu et al., 2013). Briefly, timed pregnant CFW mice (Charles River Laboratories, Wilmington, MA; Strain code: 024) were sacrificed, and neonatal (E18) mouse embryos were harvested for hippocampal cultures. Dissected hippocampi were treated with 0.05% trypsin-EDTA for 15 min at 37°C. Dissociated cells were then plated onto glass-bottomed chambers, pretreated with poly(L-lysine) (100 μg/ml). Cultures were incubated in Neurobasal medium supplemented with extra 37.5 mM NaCl, B27 supplement (2%) and Glutamax (1%) in a humidified atmosphere of 5% CO_2 at 37°C. The neurons were fed with one-half medium volume change every five days. Neurons were infected by lentivirus encoding scFv-GFP and 24xV4-ODC with or without the 3'UTR of Arc at 1 day in vitro (DIV 1). The virus dose was varied to achieve an expression level that yield good signal-to-noise ratio for the detection of single translating polysomes. All imaging experiments were performed between DIV 14-16. Before imaging the medium was exchanged into HEPES-buffered solution (HBS) containing 20 mM HEPES pH 7.4, 119 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, and 30 mM glucose. Multiple fields of view were taken to stitch the neuronal cell body and emanating dendrites together. The distances of the polysomes within the dendrites to the cell body were measured using NeuroJ (Meijering et al., 2004), a plugin for ImageJ.

Western blotting

HeLa cells plated in six-well dishes were treated by DTT (1mM) or $NaAsO_2$ (0.5mM) for various time durations as indicated, and then lysed with RIPA buffer containing protease and phosphatase inhibitor cocktail (ThermoFisher) on ice for 10 min. The lysates were collected and centrifuged at 14,000 g for 10 min. The supernatant was subjected to SDS-PAGE and transferred to nitrocellulose membrane. Following blocking in 5% (w/v) non-fat dry milk for 1 h at room temperature, primary antibodies anti-eIF2α S52P (abcam # 32157) or anti-eIF2α (cell signaling #9722) were added at 1:500 dilution for 1 h. Then, membranes were washed three times with PBS supplemented with 0.1% Tween 20 and incubated with secondary antibody conjugated to alkaline phosphatase for 1h. After washing three times with PBS supplemented with 0.1% Tween 20, the blots were developed by using SIGMA*FAST* reagent (Sigma). The densitometry of the gel image was performed using ImageJ software.

Data analysis

Translation signal measurement during drug or stress treatments

Every 5 frames of the 10-Hz movies were averaged to create 2-Hz movies. Each frame was fitted using a previously described peak-finding algorithm (Bates et al., 2007) to identify individual fluorescent foci and determine the centroid positions of these foci. Fluorescent spots identified in different frames were joined to form trajectories if the centroid positions of the spots were within 500 nm between subsequent frames. Trajectories that lasted three frames or longer were kept for subsequent analysis. To calculate the total translation activity, we determined intensities of individual translation foci and computed the total intensity of the detected translation foci in each field of view. For Puro-treatment experiments, the translation activity was sampled at a 2-Hz frequency continuously. For Homo-treatment experiments, the translation activity was recorded for 2 sec (4 frames of 2-Hz movies) in every 10 sec for a total period of 500 sec. For stress response studies, the translation activity was measured similar to Homo treatments except that the data was recorded for 2 sec (4 frames of 2-Hz movies) in every 20 sec during a 1000-sec total period.

Polysome tracking

To determine the movement properties of individual polysomes, each frame of a 10-Hz movie was fitted to identify individual fluorescent foci and determine the centroid positions of these foci. Spots within 500 nm between subsequent frames were joined to create a trajectory. Only trajectories longer than 15 frames were used for mobility analysis, while a gap of one step during tracking is allowed. Mean square distance (MSD) as a function of time delay ($n\Delta t$) was calculated by

$$\mathrm{MSD}(n\Delta t) = \frac{1}{N-n-1}\sum_{i=1}^{N-n-1} |\boldsymbol{r}(i\Delta t + n\Delta t) - \boldsymbol{r}(i\Delta t)|^2$$

where Δt is 0.1 s for the 10-Hz movies, n is the number for frames elapsed during the time delay, N is the total number of frames of the trajectory, $\boldsymbol{r}$ is the two-dimensional coordinate of the spot at each frame. To calculate the average diffusion coefficients of polysomes for a cell, the MSDs for all detected traces were averaged for the time delays of n = 1 to 10. This averaged MSD as a function of n was fit linearly, the diffusion coefficient D was determined from the slope based on the equation $\mathrm{MSD}(t) = 4Dt$.

We classified the movement of polysomes into 4 categories. If all MSD values ($n > 1$) of a trace did not deviate from the MSD at the initial $n = 1$ time point in a statistically significant manner (95% confidence interval), the trace was categorized as being stationary. If trace showed consistent movement in one direction for 1.5 μm or longer distance, the trace was categorized as directed motion. For the rest of traces, exponent α for the relationship between MSD and t, $MSD \propto t^{\alpha}$, is obtained by linear fitting of log(MSD) and log(t). For an ideal freely diffusing trace, α should be 1, whereas sub-diffusion should have an α value less than 1. Considering the error in determining α, we defined traces with α < 0.7 as being sub-diffusive. Others are diffusive. We note that by this classification, some of the super-diffusive trajectories were also characterized as being diffusive. However, since the same classification criteria were used for comparison under different conditions, the comparison can be considered largely unbiased.

For analysis of polysome mobility at perinuclear vs. non-perinuclear region, a spatial overlap with the nucleus signal, labeled by the nucleus-localized tdPCP-tdTomato was used to define the perinuclear region. This means we included those polysomes outside the nucleus but vertically aligned with the nucleus as being in the perinuclear region. This strategy is not exactly precise, but provides an operational definition to reveal the systematic difference in mobility distributions between polysomes that are near the nucleus and those that are not.

Supplemental References

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